Translation initiation by the c-myc mRNA internal ribosome entry sequence and the poly(A) tail

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ABSTRACT

Eukaryotic mRNAs possess a poly(A) tail that enhances translation via the ^{7}mGpppN cap structure or internal ribosome entry sequences (IRESs). Here we address the question of how cellular IRESs recruit the ribosome and how recruitment is augmented by the poly(A) tail. We show that the poly(A) tail enhances 48S complex assembly by the c-myc IRES. Remarkably, this process is independent of the poly(A) binding protein (PABP). Purification of native 48S initiation complexes assembled on c-myc IRES mRNAs and quantitative label-free analysis by liquid chromatography and mass spectrometry directly identify eIFs 2, 3, 4A, 4B, 4GI, and 5 as components of the c-myc IRES 48S initiation complex. Our results demonstrate for the first time that the poly(A) tail augments the initiation step of cellular IRES-driven translation and implicate a distinct subset of translation initiation factors in this process. The mechanistic distinctions from cap-dependent translation may allow specific translational control of the c-myc mRNA and possibly other cellular mRNAs that initiate translation via IRESs.

Keywords: translation initiation; cellular IRES; poly(A) tail; quantitative mass spectrometry; native RNPs

INTRODUCTION

Most eukaryotic mRNAs initiate translation via the 5′ m^{7}GpppN cap structure and the 3′ poly(A) tail (Gallie 1991; Tarun and Sachs 1995; Preiss and Hentze 1998; Gingras et al. 1999). A bridging complex between the cap-binding protein eIF4E, the adaptor protein eIF4G (I or II), and PABP bound to the 3′ poly(A) tail can mediate circularization of the mRNA (Wells et al. 1998). Biochemically, multiple mechanisms may contribute to enhance translation by: (1) promoting small ribosomal subunit recruitment via the 5′-cap structure (Tarun and Sachs 1995; Kahvejian et al. 2005); (2) stimulating 60S ribosomal subunit joining (Sachs and Davis 1989; Munroe and Jacobson 1990; Searfoss et al. 2001); and/or (3) facilitating translation termination and ribosome recycling (Uchida et al. 2002).

Translation can also initiate in a 5′-end-independent way mediated by internal ribosomal entry sequences (IRESs). IRESs are *cis*-acting RNA sequences first discovered in picornaviral RNAs (Jang et al. 1988; Pelletier and Sonenberg 1988) and also found in the 5′-untranslated region (UTR) of some cellular mRNAs (Sarnow 1989; Hellen and Sarnow 2001; Gilbert et al. 2007). Viral IRESs mediate translation by multiple mechanisms that differ in their requirements for translation initiation factors. In encephalomyocarditis virus (EMCV) IRES-mediated translation, an interaction of eIF4G and eIF4A with the IRES, in concert with the canonical eIFs 2, 3, and 4B, is necessary to recruit 40S ribosomes to the IRES. The hepatitis C virus (HCV) IRES depends on the ternary eIF2/GTP/initiator tRNA complex and on eIF3 for 48S complex formation, but does not require any eIF4 family members (Hellen and Sarnow 2001). Most remarkably, the cricket paralysis virus (CrPV) IRES can assemble 80S ribosomes without any eIFs (Wilson et al. 2000). In addition to the distinctive requirements for canonical initiation factors, noncanonical initiation factors, known as IRES *trans*-acting factors (ITAFs), have been implicated in enhancing initiation of several viral IRESs (Dorner et al. 1984; Jackson and Kaminski 1995; Kolupaeva et al. 2007). The poly(A) tail also stimulates translation via some viral IRESs (polio-, EMCV-, coxackievirus [CV] and hepatitis A virus [HAV]) (Bergamini et al. 2000; Khaleghpour et al. 2001; Michel et al. 2001; Svitkin et al. 2001; Bradrick et al. 2007). Similar to cap-dependent translation, the stimulatory effect of the poly(A) tail on viral IRES-driven translation appears to involve the eIF4G-PABP interaction (Michel et al. 2001; Svitkin et al. 2001). Numerous cellular IRESs allow protein synthesis under conditions when cap-dependent

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translation is compromised, for example during apoptosis, mitosis, and different forms of stress (Cornelis et al. 2000; Pyronnet et al. 2000; Stoneley et al. 2000; Fernandez et al. 2001; Subkhankulova et al. 2001; Kim et al. 2003; Braunstein et al. 2007; Gilbert et al. 2007) and some 3% of the cellular transcriptome continues to be translated when cap-dependent translation is inhibited by poliovirus infection (Johannes and Sarnow 1998). In several cases cellular IRES-driven translation has been attributed to the activity of ITAFs (Pilipenko et al. 2000; Stoneley and Willis 2004; Cobbold et al. 2008). Their molecular mode of action remains to be clearly defined.

The transcript encoding the c-Myc protein belongs to the mRNAs that are actively translated during apoptosis, mitosis, or cellular stress when cap-dependent translation is compromised (Stoneley et al. 2000; Subkhankulova et al. 2001; Kim et al. 2003). The expression of c-myc is critical for cell proliferation/differentiation and apoptosis (Evan and Littlewood 1998; Eisenman 2001), and the c-myc IRES has been implicated in cancer: Increased c-myc expression in cells derived from patients suffering from multiple myeloma has been attributed to somatic mutations within the IRES (Chappell et al. 2000).

In contrast to viral IRESs, very little is known about the mechanism of cellular IRES function. In particular, it is presently not understood how cellular IRESs recruit the ribosome. Using a cell-free translation system derived from HeLa cells and an in vivo assay based on RNA transfections, we showed that the poly(A) tail enhances IRES-directed translation of the c-myc and BiP mRNAs in vitro and in vivo (Thoma et al. 2004a). Here, we address the mechanistic basis for this enhancement. We show that the poly(A) tail stimulates 48S initiation complex assembly via the c-myc IRES. Remarkably, the PABP/eIF4G interaction is dispensable for this enhancement, and the enhancer function of the poly(A) tail is therefore distinct from its role in the activation of cap-dependent translation initiation. Direct purification and quantitative analysis of native c-myc IRES 48S initiation complexes identifies a distinct subset of translation initiation factors that includes eIF4GI but lacks eIF4GII.

RESULTS

PABP and intact eIF4G are dispensable for the poly(A) tail-mediated enhancement of c-myc IRES-driven translation

To examine the mechanism of translation initiation by cellular IRESs, we developed a HeLa cell-derived in vitro translation system that supports cellular IRES function (Thoma et al. 2004a,b). We found that the poly(A) tail enhances cellular (c-myc and BiP) IRES-mediated translation and showed that—in contrast to cap-dependent translation—the stimulatory effect of the poly(A) tail on cellular IRESs requires neither PABP nor intact eIF4G (Fig. 1; Thoma et al. 2004a). PABP has been proposed to stimulate cap-dependent translation initiation and ribosomal recycling (Uchida et al. 2002; Kahvejian et al. 2005). There is no information regarding the step of cellular IRES-driven translation that is affected by the poly(A) tail.

Since the c-myc IRES is active in apoptotic cells and apoptosis causes cleavage of both eIF4G I and II, we decided to study the c-myc IRES and the enhancer function of the poly(A) tail in HeLa extracts where the eIF4Gs were proteolytically cleaved (Marissen and Lloyd 1998; Bushell et al. 1999; Stoneley et al. 2000). In addition, we wanted to directly evaluate the role of PABP, as earlier studies conducted in the presence of intact eIF4G indicated that PABP was dispensable for c-myc IRES enhancement by the poly(A) tail (Thoma et al. 2004a).

PABP was depleted from the translation extracts by PAIP2-mediated affinity chromatography (Svitkin and Sonenberg 2004) by more than 90%. (Fig. 1A, right panel). To functionally confirm the effectiveness of both proteolytic eIF4G cleavage and PABP depletion, we evaluated the translation of ^{7}mGpppG-capped mRNAs with or without an poly(A) tail. Figure 1C shows that eIF4G cleavage (Fig. 1C, lanes 5,6), PABP-depletion (Fig. 1C, lanes 3,4), and the combination of the two (Fig. 1C, lanes 7,8) strongly inhibit the stimulation of cap-dependent translation by the poly(A) tail (Fig. 1C, cf. lanes 1 and 2). We then evaluated the function of PABP in the poly(A) enhancement of c-myc IRES-driven translation when eIF4G is cleaved. In sharp contrast to cap-dependent translation, c-myc IRES-driven translation is fully enhanced by the poly(A) tail, even following eIF4G cleavage and PABP-depletion (Fig. 1B, cf. lanes 7,8 and 5,6). PABP depletion even stimulates c-myc IRES activity in these extracts (Fig. 1B, cf. lanes 6 and 8). These results show that the poly(A) tail enhances c-myc IRES translation independently of PABP and intact eIF4G.

The poly(A) tail enhances translation initiation

Next, we aimed to identify the mechanism underlying this enhancer function of the poly(A) tail. Initiation is frequently rate limiting and targeted by multiple control mechanisms. To investigate whether the initiation step of c-myc IRES translation is augmented by the poly(A) tail, we analyzed ribosome assembly on c-myc mRNAs in sucrose density gradient experiments. To optimize resolution, we created a shortened form of the c-myc IRES reporter mRNA by replacing the luciferase open reading frame (ORF) with a synthetic short ORF (Fig. 2A; Gebauer et al. 2003). We validated that this shortened c-myc reporter mRNA is also translated via its IRES (Fig. 2B) and that translation of the c-myc IRES/short ORF transcripts is enhanced by the presence of a poly(A) tail (Fig. 2B).

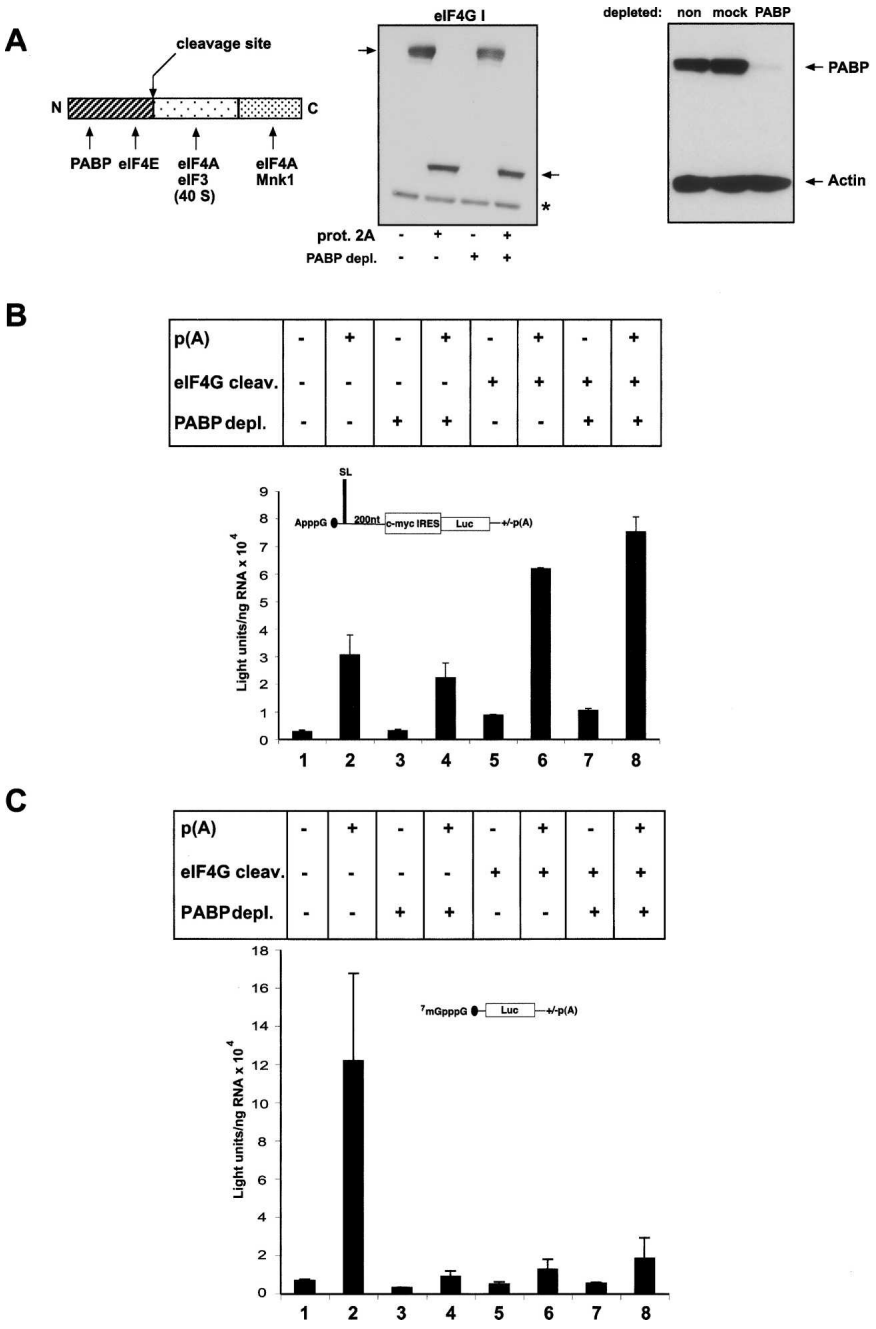


FIGURE 1. Enhancement of c-myc IRES-mediated translation is independent of intact eIF4G and PABP. (*A*) Domain structure of eIF4G with protein binding sites and the protease 2A cleavage site indicated by arrows (*left* panel). Western blot analysis of eIF4GI in the indicated extracts (*middle* panel) and PABP in non-, mock, or PABP-depleted HeLa extracts (*right* panel). The positions of intact proteins and the respective cleavage products are indicated by arrows. Asterisk denotes cross-reactive nonspecific bands. (*B*,*C*) The indicated mRNAs were translated in mock-, PABP-depleted, protease 2A-treated, or PABP-depleted and protease 2A-treated HeLa cell extracts. The data represent the average of three experiments with error bars indicating the standard deviation from the mean.

Translation initiation reactions were performed in the presence of the translation elongation inhibitor cycloheximide, which limits translation initiation exclusively to single rounds, thus excluding potential secondary effects caused by ribosome recycling. As expected, ribosomal 80S complexes assemble on c-myc IRES mRNAs (Fig. 2C, fractions 3–5, blue line). By contrast, 80S peak formation is almost absent on c-myc IRES mRNAs lacking the poly(A) tail, demonstrating directly that the poly(A) tail stimulates 80S ribosomal complex formation and hence translation initiation on c-myc IRES mRNAs (Fig. 2C, fractions 3–5, cf. pink and blue lines). Changes in mRNA stability are not responsible for the observed stimulation of c-myc IRES function by the poly(A) tail (Fig. 2D). Recall that these experiments were performed in extracts following eIF4G cleavage, implying that the observed poly(A) tail enhancement of cellular IRES-mediated initiation is independent of intact eIF4G.

The poly(A) tail stimulates 48S initiation complex formation via the c-myc IRES

Multiple mechanisms contribute to how poly(A) tails stimulate cap-dependent translation, including both 48S initiation complex formation and 60S ribosomal subunit joining (Sachs and Davis 1989; Munroe and Jacobson 1990; Tarun and Sachs 1995; Searfoss et al. 2001; Kahvejian et al. 2005). To determine how the poly(A) tail stimulates c-myc IRES-directed translation initiation, we analyzed its effect on 48S complex formation. Because GTP hydrolysis is essential for 60S ribosomal subunit joining but dispensable for the earlier steps, treatment with the nonhydrolysable GTP analog GMP-PNP arrests scanning complexes at the initiator AUG codon of the mRNA (Anthony and Merrick 1992; Pestova et al. 2000).

Translation extracts were adjusted to 2.5 mM GMP-PNP following eIF4G cleavage and incubated with c-myc IRES mRNAs bearing or lacking a poly(A) tail. Initiation complexes were resolved through 5%–25% sucrose gradients to monitor 48S initiation complex formation (see Materials and Methods). While 48S complexes form on polyadenylated c-myc IRES mRNAs (Fig. 3A, fractions 29–36, blue line),

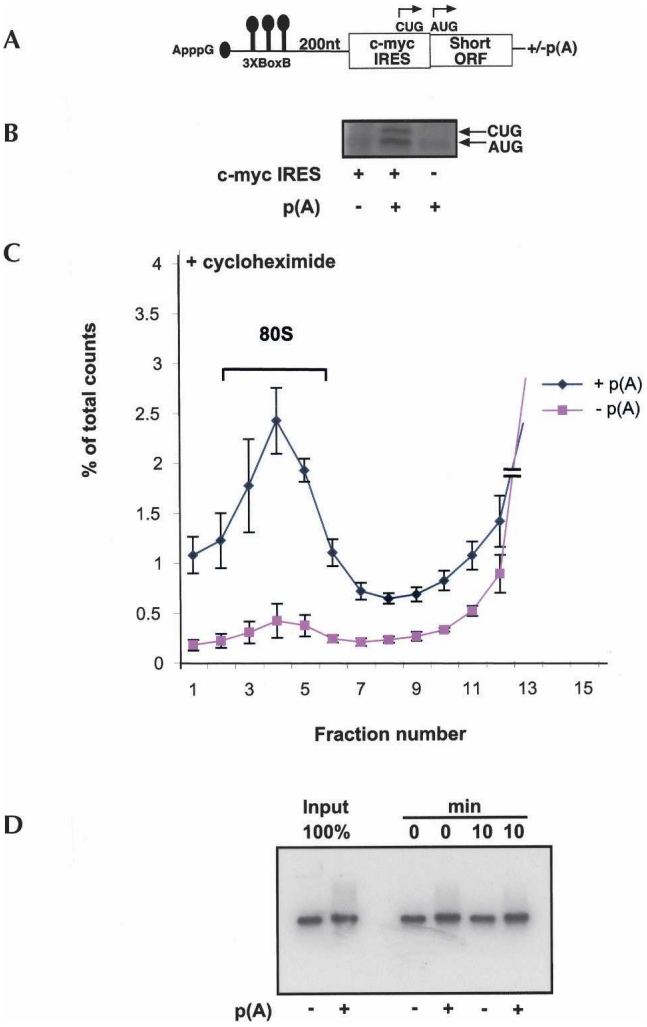


FIGURE 2. The poly(A) tail enhances 80S initiation complex assembly on c-myc IRES mRNAs. (*A*) Schematic representation of the ApppG-capped c-myc IRES-containing reporter mRNAs. Three copies of the BoxB hairpin GGGCCCT**GAAGA**AGGGCCC (loop is in bold, double strand area is underlined) were introduced upstream of a spacer sequence and the c-myc IRES for GRNA affinity chromatography (see Fig. 4). (*B*) HeLa cell extracts were programmed with ApppG-capped c-myc IRES reporter mRNAs bearing or lacking an $A_{(62)}$ tail. A reporter mRNA lacking the c-myc IRES upstream of the short ORF was used as a negative control. ^{35}S-methionine and cysteine-labeled translation products were resolved by SDS-PAGE followed by autoradiography. A representative result of at least three independent experiments is shown. (*C*) HeLa cell extracts were preincubated with coxackievirus protease 2A. Following protease treatment, in vitro translation reactions containing ^{32}P-radiolabeled ApppG-capped c-myc IRES reporter mRNAs bearing or lacking an $A_{(62)}$ tail were performed in the presence of cycloheximide. The reactions were loaded onto 5%–25% linear sucrose density gradients, and complexes were resolved by centrifugation. Fractions were taken from the bottom of the gradient and analyzed by scintillation counting. Radioactivity is expressed as percentage of total recovered counts, plotted against the fraction number. The profile of the upper fractions from the sucrose density gradients is omitted for clarity. For each mRNA, an averaged graph of three independent experiments is shown together with an indication of the experimental variability. (*D*) In vitro translation reactions containing ^{32}P-radiolabeled ApppG-capped c-myc IRES reporter mRNAs bearing or lacking an $A_{(62)}$ tail were performed in the presence of cycloheximide as in panel *C*. Total RNA was isolated before (*t*0) and after (*t*10) translation initiation and separated by gel electrophoresis followed by autoradiography.

48S complex recruitment to c-myc IRES mRNAs lacking a poly(A) tail is almost nondetectable (Fig. 3A, pink line). Taken together, these experiments show that the poly(A) tail stimulates 48S ribosomal complex formation with c-myc IRES mRNAs, apparently independently of intact eIF4G.

Enhancement of 48S ribosomal complex formation on c-myc IRES mRNA by the poly(A) tail is independent of PABP

Since we observed that the enhancement of IRES-mediated translation by the poly(A) tail is independent of PABP (Fig. 1), we wanted to examine directly whether 48S initiation complex recruitment to the c-myc IRES is also PABP independent. We depleted PABP from HeLa cell extracts such that the strong poly(A) enhancement of cap-dependent translation is almost completely lost (Fig. 1C, cf. lanes 1,2 and 3,4,7,8). PABP-depleted extracts continue to show strong poly(A) enhancement of 48S initiation complex formation with the c-myc IRES mRNA, albeit slightly less than mock-depleted extracts (Fig. 3B). Thus, PABP is dispensable for the poly(A)-mediated stimulation of 48S complex formation with c-myc IRES mRNA, in contrast to its role in cap-dependent translation.

Quantitative label-free analysis of native 48S c-myc IRES translation initiation complexes by liquid chromatography and mass spectrometry

Viral IRES elements utilize different eIFs for translation initiation. There is currently no such information available for cellular IRESs. Therefore, we set out to analyze the composition of c-myc IRES 48S translation initiation complexes assembled on the polyadenylated mRNAs. To this end, we used the three boxB RNA hairpins upstream of the IRES; as a specificity control, a c-myc IRES mRNA lacking the boxB sequences was used (Fig. 4A). The boxB-containing c-myc RNAs were validated to initiate translation as efficiently as their counterparts lacking the boxB insertion (data not shown). Native ribosomal 48S complexes were then assembled in the presence of GMP-PNP in eIF4G-cleaved and PABP-depleted HeLa cell extracts and resolved on sucrose density gradients. Nontreated extracts (i.e., no PABP depletion or eIF4G cleavage) were used as a control. 48S initiation complexes were then isolated from gradient fractions 27 to 33 by GRNA chromatography (Fig. 4A; Czaplinski et al. 2005; Duncan et al. 2006). The eluted complexes were analyzed by quantitative label-free shotgun proteomics (Fraterman et al. 2007). Importantly, this strategy allows validation of the 48S ribosomal complexes by determining the enrichment of small versus large ribosomal subunit proteins. It also permits the comparative quantitative analysis of eIF association in control versus eIF4G-cleaved and PABP-depleted extracts. In particular, it

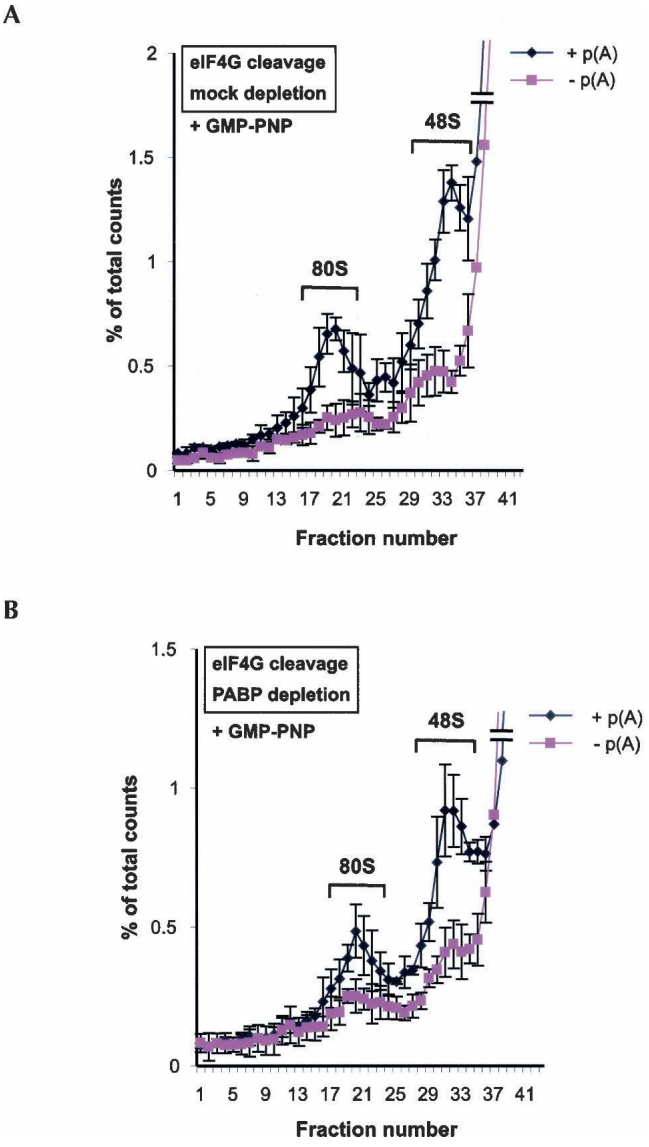


FIGURE 3. The poly(A) tail enhances 48S complex formation with c-myc IRES mRNAs in a PABP-independent manner. HeLa extracts were treated as described in Figure 2C. In vitro translation reactions containing ^{32}P-radiolabeled ApppG-capped c-myc IRES reporter mRNAs bearing or lacking an $A_{(62)}$ tail were performed in the presence of GMP-PNP in control (*A*) and PABP-depleted (*B*) extracts, and analyzed as described in Figure 2C. An additional peak forms with polyadenylated c-myc mRNAs further toward the bottom of the gradient (*A*, fractions 17–24, blue line). This minor peak may represent 80S ribosomal complexes that assemble due to leakiness from incomplete replacement of endogenous GTP by GMP-PNP. This explanation is supported by an analysis of initiation complex formation on c-myc mRNAs in extracts supplemented with either cycloheximide alone or cycloheximide plus GMP-PNP (data not shown). The profile of the upper fractions from the sucrose density gradients is omitted for clarity. For each mRNA, an averaged graph of three independent experiments is shown together with an indication of the experimental variability.

allows the quantitative analysis of PABP association with c-myc IRES translation initiation complexes from the two different extracts by comparison of ion volumes of BoxB-containing samples with the "no BoxB" control samples and calculation of a "fold increase" representing the enrichment of purified proteins.

Whereas 60S ribosomal proteins are not enriched, 40S ribosomal proteins are ninefold enriched on BoxB-containing mRNAs over the "no BoxB" controls (Fig. 4B), showing that 48S translation complexes are specifically purified. We next addressed the question of whether the effective stimulation of translation by the poly(A) tail in PABP-depleted extracts might be caused by highly efficient recruitment of residual PABP to the c-myc IRES mRNA. At the same time we explored the possibility of whether a PABP isoform that might have escaped depletion mediates the poly(A) effect. In humans, three cytoplasmic PABP isoforms (testis PABP, inducible PABP, and PABPC5) have been identified in addition to the well-studied major isoform PABPC1 (Gorgoni and Gray 2004). Although two PABP isoforms, PABPC1 and PABPC4, are identified in c-myc IRES 48S initiation complexes, recruitment of both PABPC1 and PABPC4 to polyadenylated c-myc IRES mRNA is almost abolished in eIF4G-cleaved and PABP-depleted extracts (Fig. 5A). This result implies that PABP is not a mediator of the poly(A) enhancement of cellular IRES translation. It also implicates a distinct factor(s) in the poly(A)-mediated translational stimulation of cellular IRESs.

The functions of eIF4GI and eIF4GII in translation initiation were initially considered to be largly redundant (Goyer et al. 1993; Gradi et al. 1998). Our direct analyses now identify eIF4A and eIF4GI as specific components of native 48S initiation complexes (Fig. 5B). These complexes appear not to contain eIF4GII (Fig. 5B), although eIF4GII is abundant in HeLa cell extracts (Hundsdoerfer et al. 2005). The abundance of eIF4GI and eIF4GII previously has been determined to be 3 pmol/μL HeLa cell extract for eIF4GI and 4–5 pmol/μL HeLa cell extract for eIF4GII (Hundsdoerfer et al. 2005). We have confirmed these quantitative data for the HeLa extracts used in this study (data not shown).

Note that eIF4GI, eIF4A, and eIF4B levels are reduced 4.9-fold, 2.9-fold, and 1.9-fold, respectively, in eIF4G-cleaved and PABP-depleted extracts compared to control extracts (Fig. 5B). This result is not a technical artifact arising from eIF4G cleavage, because the method reliably detects peptides from all regions of eIF4GI (Fig. 5C). The native IRES 48S initiation complexes also contain the translation initiation factors eIF2, eIF3, and eIF5. The levels of these eIFs vary only between 1.1-fold for eIF3 and 1.4-fold for eIF5, respectively, in eIF4G-cleaved and PABP-depleted versus control extracts. Since both extracts display active IRES-mediated translation, these results directly implicate these initiation factors in the c-myc IRES-driven translation initiation mechanism.

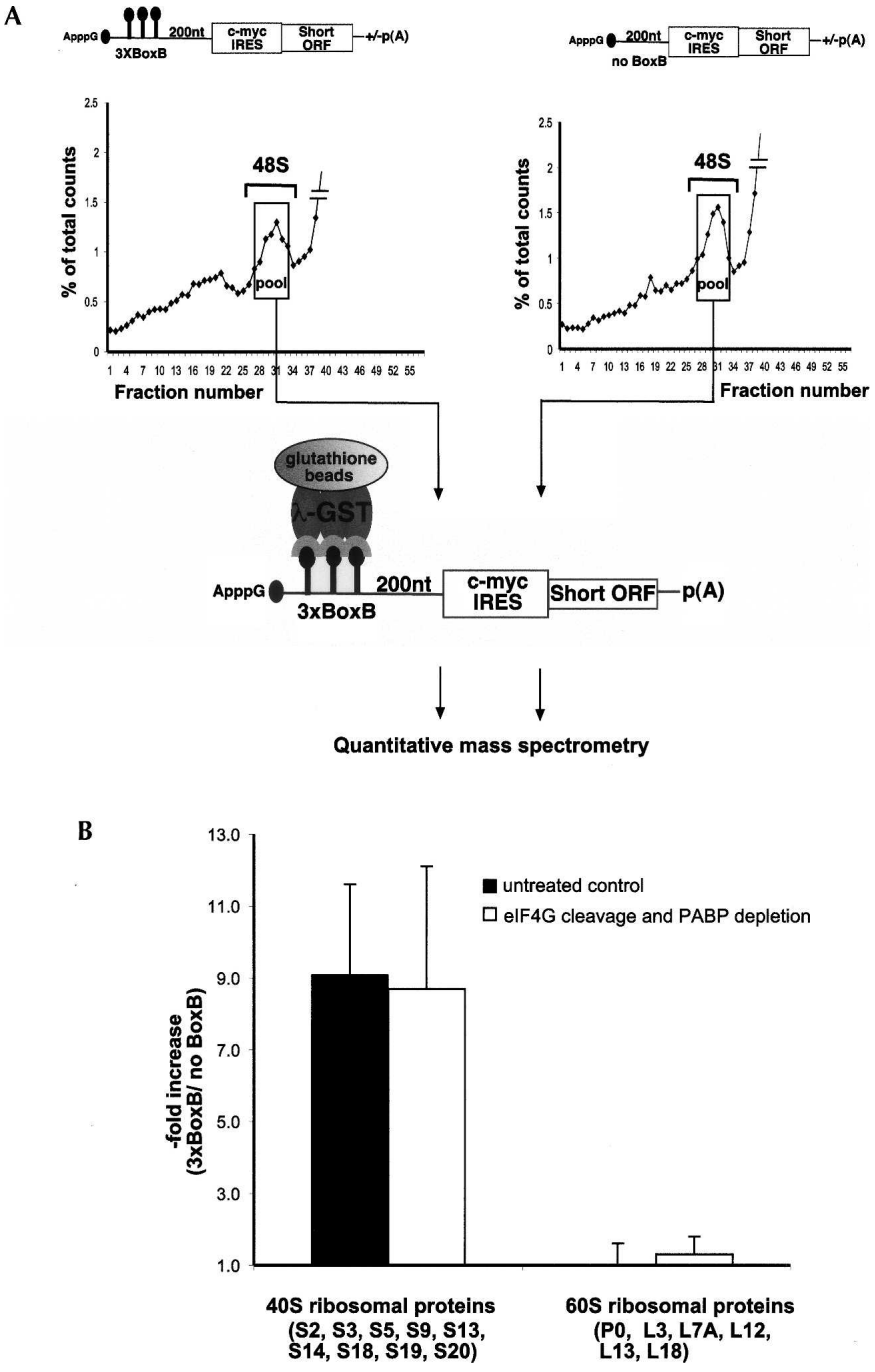


FIGURE 4. Isolation and characterization of native c-myc IRES 48S initiation complexes. (*A*) A schematic representation of ApppG-capped c-myc IRES-containing reporter mRNAs used for GRNA affinity chromatography via the lambda phage N-protein/boxB hairpin interaction is shown *above* the purification scheme. Purifications were performed from control extracts and PABP-depleted extracts that were treated with coxackievirus protease 2A. Translation reactions were programmed with the indicated ^{32}P-radiolabeled ApppG-capped mRNAs in the presence of GMP-PNP. The reactions were subjected to 5%–25% linear sucrose density gradient analysis; 48S fractions were pooled prior to isolation by GRNA chromatography. The gradient profile of 3xboxB-containing c-myc mRNAs (*left* panel) and the negative control without the boxB elements (*right* panel) incubated in eIF4G-cleaved and PABP-depleted reactions is shown as an example. The eluates from the GRNA affinity chromatography were analyzed by quantitative mass spectrometry. (*B*) Quantitative mass spectrometry analysis of c-myc IRES translation initiation intermediates derived from control versus eIF4G-cleaved and PABP-depleted extracts. Calculated fold changes result from the comparison of 3xboxB-containing c-myc mRNAs with the no boxB control. Error bars for 40S and 60S ribosomal proteins were calculated based on the fold changes of individual ribosomal proteins identified in the duplicate analyses.

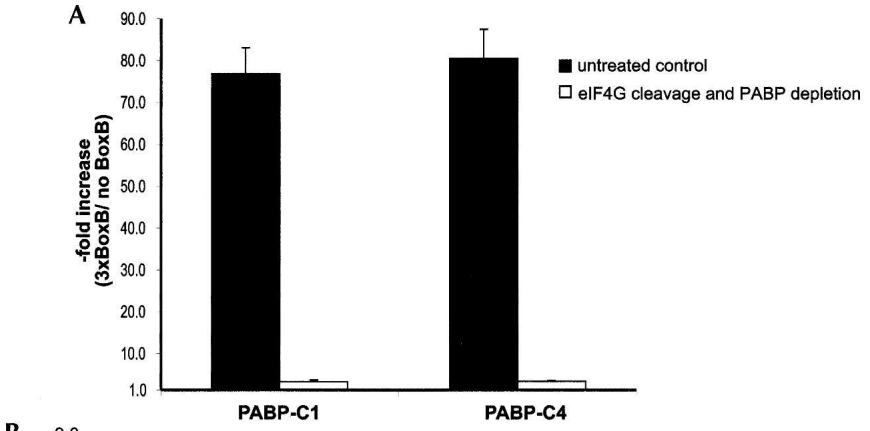
DISCUSSION

The poly(A) tail is essential for c-myc IRES-driven translation in vivo (Thoma et al. 2004a). Here, we describe the underlying mechanism using a validated HeLa cell-derived in vitro translation system (Bergamini et al. 2000; Thoma et al. 2004a,b; Hundsdoerfer et al. 2005). The data reveal that the poly(A) tail enhances first round translation initiation by the c-myc IRES. Specifically, it augments 48S complex recruitment and does so in a way that is distinct from its activation of cap-dependent translation initiation, independently of intact eIF4G and PABP. Analysis of native 48S initiation complexes by quantitative mass spectrometry yields direct information on the composition of these particles, including the differential association of eIF4GI in comparison to eIF4GII.

The question of how the poly(A) tail enhances translation via a cellular IRES has not yet been addressed. We show that 80S complex assembly is stimulated by the poly(A) tail on c-myc IRES mRNA (Fig. 2C); hence, the poly(A) tail enhances translation initiation. The quantitatively strong effect of the poly (A) tail on 80S complex formation suggests that this is a major contribution to the overall translational effect (Fig. 1B). A potential additional contribution to a post-initiation step (i.e., elongation, termination) or to ribosome recycling, however, is not excluded by these data.

The marked enhancement of 48S ribosome complex formation with c-myc mRNA (Fig. 3) further reveals that the poly(A) tail promotes translation initiation at an early step by increasing the stable binding of 48S initiation complexes to the mRNA. Since it is unknown whether 80S complexes and 48S complexes are equally stable in sucrose gradient analyses, direct quantitative comparisons are difficult. Nonetheless, the poly(A) tail enhancement of both steps does not appear to differ dramatically, indicating that 60S subunit joining is not a primary target.

What are the molecular requirements for this enhancer function? Intact eIF4G,



C

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*GGK**DITEEIM SGAR**TASTPT PPQTGGGLEP QANGETPQVA VIVRPDDRSQ 151*
GAIIADRPGL PGPEHSPSES QPSSPSPTPS PSPVLEPGSE PNLAVLSIPG 201
DTMTTIQMSV EESTPISRET GEPYRLSPEP TPLAEPILEV EVTLSKPVPE 251
SEFSSSPLQA PTPLASHTVE IHEPNGMVPS EDLEPEVESS PELAPPPACP 301
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*DVVLDKANKT PLRPLDPTR**L QGINCGPDFT PSFANLGR**TT LSTRGPPRGG 601*
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IDTEERLKGV IDLIFEKAIS EPNFSVAYAN MCR**CLMALK**V PTTEKPTVTV 751
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RDIARRRSLG NIKF**IGELFK** LKMLTEAIMH DCVVKLLKNH DEESLECLCR 851
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KEVEERSRER PSQPEGLRKA ASLTEDRDRG RDAVKREAAL PPVSPLK**AAL** 1151
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WLREAEEESD HN

FIGURE 5. Translation factors associated with native c-myc IRES 48S initiation complexes assembled in control versus eIF4G-cleaved and PABP-depleted extracts. Calculated fold changes result from the comparison of 3x boxB-containing mRNAs with the no boxB control. (*A*) Both PABPC1 and PABPC4 levels are 26-fold reduced after PABP depletion. Error bars are calculated based on the individual fold changes of each replicate. (*B*) Error bars for eIF2 and eIF3 were calculated based on the fold changes of individual subunits identified in the repeated analysis. Error bars for the other initiation factors were calculated based on the duplicate analysis. Peptides for eIF4A and eIF4B were only identified in one of the two replicates due to undersampling of the mass spectrometer. Therefore error bars are missing. eIF4E, eIF1, eIF1A, and eIF5B were not detected. (*C*) Primary structure of eIF4GI. The N-terminal domain is presented in italics. Peptides identified by mass spectrometry derived from control extracts are indicated in bold.

which is critical for cap-dependent translation (Liebig et al. 1993), is dispensable for the stimulatory poly(A) effect on 48S complex assembly on c-myc IRES mRNA (Fig. 3A). This result explains previous work showing that the enhancement of cellular IRES-driven translation by the poly(A) tail is independent of intact eIF4G (Thoma et al. 2004a).

Isolation of native translation initiation intermediates and determination of their composition could shed light on the involvement of translation initiation factors in c-myc IRES translation. To this end, we combined GRNA chromatography (Czaplinski et al. 2005; Duncan et al. 2006) with quantitative label-free shotgun mass spectrometry (Fraterman et al. 2007). Interestingly, we specifically identify eIF4GI but not eIF4GII within c-myc IRES-containing 48S complexes (Fig. 5B), even though eIF4GII is as abundant as eIF4GI in our HeLa cell extracts (Hundsdoerfer et al. 2005; data not shown). Hence the two isoforms of eIF4G are not functionally redundant, at least in c-myc IRES translation. However, we cannot formally exclude the possibility that eIF4GII may have dissociated during the purification procedure.

We observe a reduced association of eIF4GI, eIF4A, and eIF4B with c-myc IRES 48S translation complexes purified from eIF4G-cleaved and PABP-depleted extracts (Fig. 5B). This is unexpected and interesting, given the importance of eIF4GI and eIF4A in c-myc IRES-driven translation (Thoma et al. 2004a; Hundsdoerfer et al. 2005). Possibly, binding of eIF4GI to the c-myc IRES-containing mRNA is weakened after cleavage, which would destabilize associated factors. It has been proposed for ^{7}mGpppG-capped mRNAs that PABP increases the affinity of eIF4G for the mRNA via its interaction with the N terminus of eIF4G. In this case, disruption of the eIF4G/PABP interaction by eIF4G cleavage and/or PABP depletion may well affect the stability of eIF4G binding. Work on EMCV IRES-mediated initiation shows that eIF4A functions as part of a complex

with eIF4G rather than as a singular protein (Hellen and Sarnow 2001). This may also explain the concomitant reduction of eIF4A association in our experiments. The drop of eIF4B might be a consequence of PABP depletion, as it has been shown that eIF4B interacts with PABP (Bushell et al. 2001). This interpretation points to the technical shortcoming of our approach: Weakly associated factors will likely be lost from ribosomal complexes before affinity purification and proteomic analysis because of the stringency of the sucrose density gradient step. This may account for the failure to identify eIF1 or eIF1A in the c-myc IRES-containing 48S complexes (Fig. 5B). Other initiation factors (e.g., eIF 2, 3, and 5) and ribosomal proteins are not reduced (Figs. 4B, 5B), excluding differences in the purification procedure as the underlying cause for the effects on eIF4GI, eIF4A, and eIF4B. An interesting possibility that may also explain these results is that eIF4GI, eIF4A, and eIF4B may exert their function *prior* to the completion of 48S complex assembly and then dissociate. In this scenario, their continued association with the c-myc IRES mRNAs in control extracts may reflect a stabilizing function of PABP. Taken together, the proteomic analysis of the c-myc IRES initiation intermediates further supports the notion that the poly(A) enhancement of 48S complex assembly on c-myc IRES mRNA is unaffected by eIF4GI cleavage. This may explain why the c-myc IRES remains translationally active during important physiological processes when eIF4G is cleaved and cap-dependent translation is compromised during, for example, apoptosis, mitosis, and stress.

We also explored the role of PABP by the proteomic approach. Following PABP depletion from the translation extracts, both PABPC1 and PABPC4 association with polyadenylated c-myc IRES mRNA drastically is reduced (Fig. 5A), although c-myc IRES translation is even 2.5-fold increased (Fig. 1B). This result directly argues against the possibility that residual PABP is more effectively recruited to c-myc IRES containing mRNAs. How does the poly(A) tail enhance c-myc IRES-driven translation initiation? Analogous to the eIF4E–eIF4G–PABP bridging complex that enhances cap-dependent translation, the poly(A) tail and the c-myc IRES may be similarly bridged. It is also possible that the poly(A) tail acts by changing the mRNA secondary structure and that such a structural change contributes to enhanced (PABP-independent) c-myc IRES translation initiation. Future experiments will aim to distinguish between these possibilities.

MATERIALS AND METHODS

Plasmids

The pT3luc(pA) and pSL200Mluc(pA) plasmids have been described (Iizuka et al. 1994; Hundsdoerfer et al. 2005). To generate pBbox200MS(pA), a NcoI/SpeI fragment containing the luc ORF of pSL200Mluc(pA) was replaced with a NcoI/HpaI fragment of the plasmid pBSEF containing a synthetic short ORF (Gebauer et al. 2003) generating pSL200MS(pA). Next, three BoxB sequences derived from pBSEF-BoxB (Duncan et al. 2006) were introduced into the KpnI site of pSL200MS(pA). To obtain the "no IRES" control construct, the HindIII/NcoI fragment of Bbox200MS(pA) containg the c-myc IRES was removed. All constructs were verified by DNA sequencing. The plasmid pλGST for GRNA chromatography was kindly provided by K. Czaplinski (Czaplinski et al. 2005).

In vitro transcription and translation

In vitro transcription of mRNAs in the presence of either ^{7}mGpppG or ApppG, the preparation of HeLa cell extracts, and in vitro translation assays were described previously (Bergamini et al. 2000). We used 1.5 mM MgOAc and 60 mM KOAc in translation reactions. All reactions were performed in micrococcal nuclease-treated HeLa extracts. Micrococcal nuclease treatment and protease 2A treatment of HeLa cell extracts were described previously (Thoma et al. 2004a). The concentration of exogenous mRNA was 1 ng/μL for ^{7}mGpppG-capped luc mRNAs, 5 ng/μL for ApppG-capped c-myc IRES luc mRNAs, and 15 ng/μL for c-myc IRES short ORF mRNAs. Translation reactions were incubated at 37°C for 30 min. For labeling of proteins, [^{35}S] methionine (0.75 mCi/mL) and cysteine (1.43 mCi/mL) were substituted for unlabeled methionine and cysteine in the translation reaction.

PABP depletion from HeLa cell extracts

Affinity depletion of PABP using immobilized PAIP2 was performed as described (Svitkin and Sonenberg 2004; Thoma et al. 2004a).

Recombinant proteins

Coxackievirus protease 2A was generously provided by the late Ernst Kuechler (Vienna, Austria). λ-GST protein was expressed in *Escherichia coli* and purified as described (Czaplinski et al. 2005).

Western blotting and antibodies

The following antibodies were used: monoclonal anti-eIF4GI antibody, 1:250 dilution (BD Transduction Laboratories, Pharmingen), monoclonal anti-PABP antibody 10E10, 1:3000 dilution (kindly provided by M. Görlach, Jena, Germany), and monoclonal anti-actin antibody, 1:5000 dilution (Sigma). Samples were separated by SDS-PAGE and electroblotted onto PVDF membranes (Immobilon, Millipore). Protein signals were detected using enhanced chemiluminescent procedure.

Sucrose density gradient analysis

Translation initiation intermediates were assembled on radiolabeled mRNAs in 50-μL reactions. We used 1 mM cycloheximide or 2.5 mM GMP-PNP to stall defined initiation intermediates. Reactions were incubated for 10 min at 37°C, then diluted 1:1

with sucrose gradient buffer (16 mM HEPES at pH 7.4, 60 mM KAc, 1.5 mM MgAc, 1 mM DTT), and loaded on top of linear 5%–25% sucrose density gradients (4.5 mL for the separation of 80S complexes and 10 mL for the separation of 48S complexes). After centrifugation at 30,000 rpm for 3 h at 4°C in a SW55TI rotor (80S) or 38,000 rpm for 3 h at 4°C in a SW40 rotor (48S), respectively, fractions were collected from the bottom of the gradient and analyzed by scintillation counting.

GRNA chromatography

For native initiation complex purifications, we adapted a previously described protocol (Duncan et al. 2006). For each RNA, five separate reactions were used for sucrose gradient purifications and later pooled for mass spectrometry analysis. For each BoxB-containing c-myc IRES mRNA, an amount corresponding to 4 pmol was subjected to elution after purification. For each individual reaction, 45 pmol of RNAs were incubated in 500 μL of in vitro translation reactions for 10 min at 37°C and subsequently loaded onto a 10 mL linear sucrose density gradient. After centrifugation at 38,000 rpm for 3 h, fractions were collected from the bottom. 48S fractions (Fig. 4A) of each gradient were pooled. One milliliter of pooled fractions was supplemented with heparin, NP-40, and glycerol to a final concentration of 12 μg/mL, 0.05%, 8.7%, respectively, and added to 60 μL of glutathione-Sepharose (Amersham) prebound to 35 μg λ-GST protein. Samples were incubated for 3 h with end-over-end rotation, then washed three times with 400 μL of ice-cold binding buffer (Duncan et al. 2006). Counts associated with beads were measured by scintillation counting. RNA-associated proteins were eluted by adding 10 μL ice-cold binding buffer and 0.7 μL bovine pancreatic RNase A (100 μg/mL stock, protease-free, Calbiochem) to the beads and incubating at 30°C for 30 min with shaking. Eluates were precipitated with methanol and chloroform and processed by label-free quantitative mass spectrometry.

Label-free quantitative mass spectrometry

Peptides were trypsin-digested in solution and separated on a nano-flow 1D-plus Eksigent HPLC system coupled with a QStar Pulsar *i* quadrupole Time-of-Flight MS (Applied Biosystemsy) as described (Fraterman et al. 2007). Peptides were identified by searching the peak-list against the IPI human (3.19_20060712, 60,937 sequence entries) database using the MASCOT v2.103 (Matrix Science) algorithm and standard search parameters. All peptides were identified with a MASCOT score above 18. Peptide sequences were checked for isoform specificity as previously described (Fraterman et al. 2007). For each protein the number of fragment spectra matched by MASCOT is shown in Table 1.

TABLE 1. Protein identifications of GRNA chromatography eluates

Protein	Accession no.	Molecular weight	Queries matched
Polyadenylate-binding protein 1	IPI00008524	70,854	49
Polyadenylate-binding protein 4	IPI00012726	71,080	37
Eukaryotic translation initiation factor 3 a	IPI00029012	168,677	22
40S ribosomal protein S18	IPI00013296	17,708	19
Eukaryotic translation initiation factor 4 gamma 1	IPI00220365	155,450	16
40S ribosomal protein S3	IPI00011253	26,842	13
Eukaryotic translation initiation factor 3 c	IPI00016910	105,962	13
40S ribosomal protein S2	IPI00013485	31,590	12
40S ribosomal protein S9	IPI00221088	22,504	12
Eukaryotic translation initiation factor 2 β	IPI00021728	38,707	11
40S ribosomal protein S19	IPI00215780	15919	10
Eukaryotic translation initiation factor 2 γ	IPI00297982	51,516	10
40S ribosomal protein S6	IPI00021840	28,834	9
40S ribosomal protein S20	IPI00012493	13,478	7
Eukaryotic translation initiation factor 2 α	IPI00398135	16,726	7
Eukaryotic translation initiation factor 3 d	IPI00006181	64,560	6
40S ribosomal protein S13	IPI00221089	17,081	5
40S ribosomal protein S5	IPI00008433	22,902	5
Eukaryotic translation initiation factor 5	IPI00022648	49,648	5
40S ribosomal protein S14	IPI00026271	16,303	4
Eukaryotic translation initiation factor 3 i	IPI00012795	36,818	4
60S ribosomal protein P0	IPI00008530	34,423	3
60S ribosomal protein L12	IPI00024933	17,979	3
60S ribosomal protein L13	IPI00465361	24,173	3
Eukaryotic initiation factor 4A	IPI00025491	46,353	3
60S ribosomal protein L3	IPI00055021	46,234	3
Eukaryotic translation initiation factor 3 j	IPI00012795	36,878	3
60S ribosomal protein L18	IPI00026202	21,034	2
60S ribosomal protein L7a	IPI00299573	30,017	2
Eukaryotic translation initiation factor 4B	IPI00012079	69,183	2

For each protein identified in the study, protein name, accession number, molecular weight in Daltons, and the number of queries matched by MASCOT search engine are presented.

Quantitative analysis was performed using MSQuant in a no label setting (Schulze and Mann 2004). The quantitation results (peptide ion volumes in Thompson·seconds) for differentially expressed peptides were visually inspected for anomalies. To retrieve quantitative data for peptides that were only identified in a single experiment, the resulting MASCOT search result file was cross-correlated with generic mass spectrometric data from other samples (Pasa-Tolić et al. 2004). Protein ion volumes were based on the average ion volumes of two different peptide ion volumes. The presented "fold increase" is the ratio of the ion volumes of the BoxB-containing samples to the no BoxB control samples.

For eIF2, eIF3, 40S, and 60S ribosomal proteins, an average of the individual subunit protein fold increases was calculated and presented. The presented quantitative result represents the average of two independent experimental replicates. Peptides for eIF4A and eIF4B were only identified in one of the two replicates due to undersampling of the mass spectrometer and therefore only a single replicate was used to calculate the quantitative information. Standard deviations were calculated using Microsoft Excel (Microsoft) and presented as error bars (Fraterman et al. 2007).

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